In the name of god

Gold nanoparticles stabilize peptide-drug-conjugates for sustained targeted drug delivery to cancer cells

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RESEARCH

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Contact

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Gold nanoparticles stabilize peptide-drug-conjugates for sustained targeted drug delivery to cancer cells

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Abstract

Background: Peptide-drug-conjugates (PDCs) are being developed as an effective strategy to specifically deliver cytotoxic drugs to cancer cells. However one of the challenges to their successful application is the relatively low stability of peptides in the blood, liver and kidneys. Since AuNPs seem to have a longer plasma half-life than PDCs, one approach to overcoming this problem would be to conjugate the PDCs to gold nanoparticles (AuNPs), as these have demonstrated favorable physico-chemical and safety properties for drug delivery systems. We set out to test whether PEG coated-AuNPs could provide a suitable platform for the non-covalent loading of pre-formed PDCs and whether this modification would affect the bioavailability of the PDCs and their cytotoxicity toward target cancer cells.

Methods: Peptides specifically internalized by A20 murine lymphoma cells were isolated from a phage library displaying 7mer linear peptides. Peptide specificity was validated by flow cytometry and confocal microscopy. PDCs were synthesized containing a selected peptide (P4) and either chlorambucil (Chlor), melphalan (Melph) or bendamustine (Bend). Gold nanoparticles were sequentially coated with citrate, PEG-6000 and then PDC (PDC-PEG-AuNP). The physico-chemical properties of the coated particles were analyzed by electrophoresis, TEM, UV–VIS and FTIR. Stability of free and PDC-coated AuNP was determined.

Results: Biopanning of the phage library resulted in discovery of several novel peptides that internalized into A20 cells. One of these (P4) was used to synthesize PDCs containing either Chlor, Melph or Bend. All three PDCs specifically killed A20 target cells, however they had short half-lives ranging from 10.6 to 15.4 min. When coated to PEG-AuNPs, the half-lives were extended to 21.0–22.3 h. The PDC-PEG-AuNPs retained cytotoxicity towards the target cells. Moreover, whereas pre-incubation for 24 h of free PDCs almost completely abolished their cytotoxic activity, the PDC-PEG-AuNPs were still active even after 72 h pre-incubation.

Conclusions: Peptide-drug-conjugates hold potential for improving the target efficacy of chemotherapeutic drugs, however their short half-lives may limit their application. This hurdle can be overcome by easily conjugating them to gold nanoparticles. This conjugation also opens up the possibility of developing slow release formulations of targeted drug delivery systems containing PDCs.

Keywords: Targeted drug delivery, Peptide drug conjugates, Phage display, Gold nanoparticles

Background

The importance of developing of targeted drug delivery (TDD) systems:

systems are important to develop because their potential to minimize off-target side effects may lead to a significant widening of the therapeutic window. However to achieve this goal it will in part, be necessary to improve the stability of the carrier-drug constructs so as to ensure restricted release of the drug payload to the tumor cell or at least its local environment.

Background

- TDD to tumor cells strategy is based on:
  cell surface heterogeneity between normal cells and cancer cells

- Example: mitigating the selectivity problems of chemotherapy using high affinity biomolecular carriers such as antibodies

- There is a number of challenges to antibody–drug-conjugates efficient use

Background

This study focus is to use much smaller carriers such as peptides

- ❖ peptide-drug-conjugates (PDCs)

- ✓ Specific for their target cells
- ✓ Do not induce outgrowth of drug resistant cells
- ✓ Can reverse drug resistance and can deliver multi-drug payloads

One of the remaining challenges to the successful application of PDCs for cancer therapy is the relatively low stability of peptides to enzymatic hydrolysis in the blood, liver and kidneys [9]. One approach to overcoming this prob-

- ❖ Conjugate the PDC to Gold nanoparticles overcomes their low stability

Background

Gold nanoparticles (AuNPs)

- Favorable physico-chemical and safety properties
- Ease of synthesis
- Ligand modification is not necessary for chemiadsorption on to the colloidal gold surface
- Can easily be conjugated to peptides, enzymes, DNA and small molecule drugs
- Improved pharmacokinetic and pharmacodynamics properties (when used as drug delivery Systems)
- Lead to improved drug stability and reduced side effects

❖ The study hypothesize

Since AuNPs seem to have a longer plasma half-life than PDCs, we thought to use them not as a drug delivery mechanism but rather as a PDC stabilizer. We hypothesized that PDCs conjugated to AuNPs may improve the former's bioavailability. We set out to test whether PEG coated-AuNPs could provide a suitable platform for the non-covalent loading of pre-formed PDCs and whether this modification would affect the bioavailability of the PDCs and their cytotoxicity toward target cancer cells.

Methods

1-Cell culture

Normal cells (CC-2811 and CC2553)
3T3 mouse fibroblasts, human HL-60, NB4 and murine A20 leukemic cells
Mouse MOPC 315.BM cells
Murine heart and kidney cells

2-Removal of irrelevant phage clones by in vitro negative selection

3-Positive selection for A20 internalized phage peptides

4- Peptide synthesis

5- Specificity of peptide binding

6- Confocal microscopy

7- Synthesis of peptide–drug conjugates (PDC)

8- Synthesis of citrate-coated gold nanoparticles

9- PEG-6000 coated gold nanoparticles

10- Determination of loading efficiency of PDC on PEG AuNPs

11- Characterization of the PDC-PEG-AuNPs

Electrophoretic mobility assay and Morphology

12- Stability of PDCs and PDC-coated PEG-GNPs

Chemostability and Biostability in liver homogenate

13-Cell cytotoxicity assay

Results

Identification of phage peptides specifically internalized by A20 cells

Before exposure to the target cells, the stock Ph.D-7 linear phage display library was sequentially absorbed in vitro on a series of normal human and mouse cells and on Matrigel, in an effort to remove as many phage clones as possible that display peptides against normal cell surface and matrix polymer components. As shown in Additional file 1: Figure S2, this process reduced the stock concentration from ~ 3×10^{10} pfu/μl to ~ 10^6 pfu/μl. This absorbed library was then amplified to expand the number of each of the remaining clones and to restore the initial phage concentration.

The absorbed library was then exposed to A20 cells (three rounds of selection)

Identification of phage peptides specifically internalized by A20 cells

Internalized phage from cycle 3 were titrated on bacterial lawns and 15 isolated plaques were randomly selected and designated P1, P2, P3...P15. ssDNA was extracted

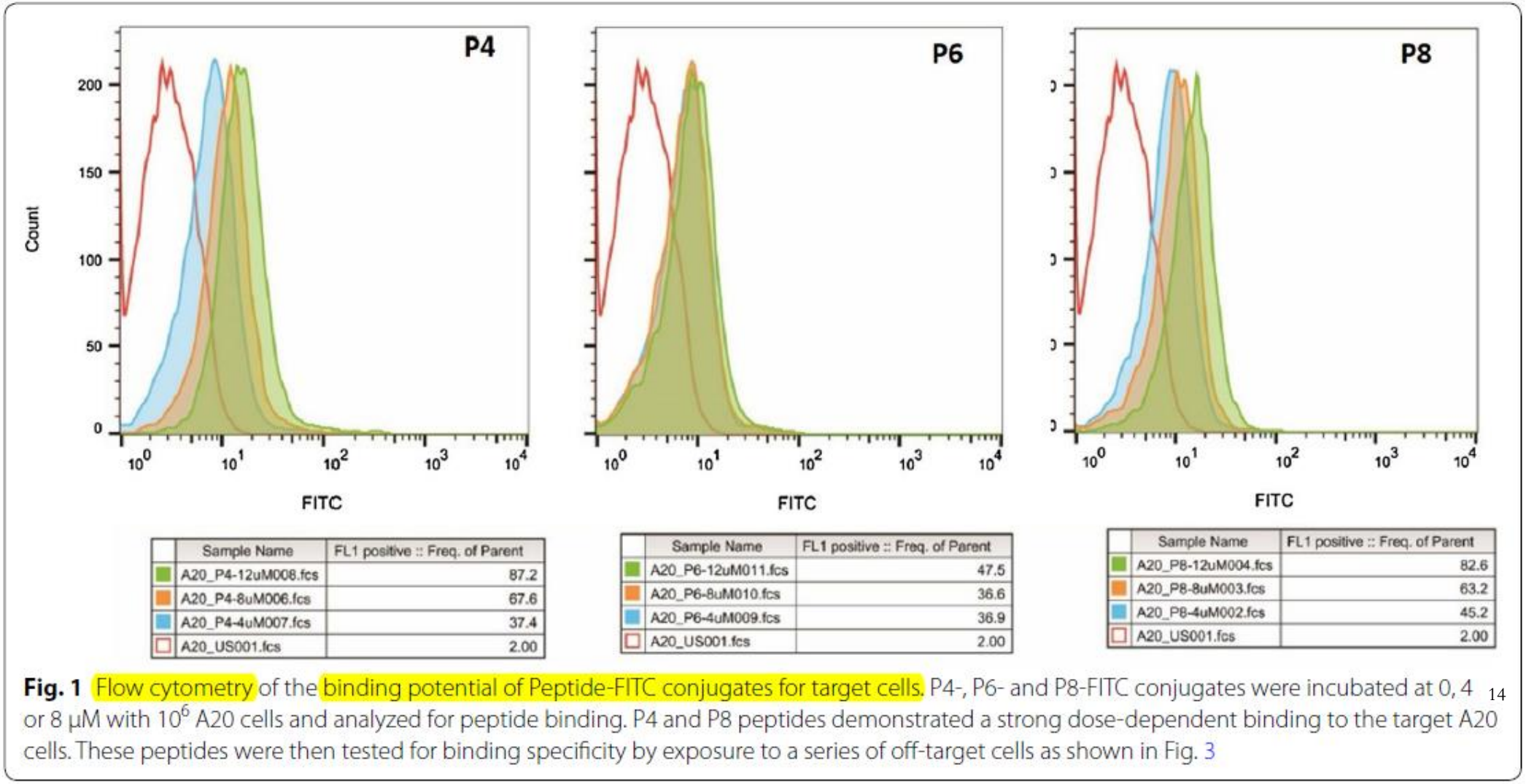
Table 1 Peptide sequences of phage internalized by A20 cells and the frequency amongst the sequenced clones

Clone designation	Peptide sequence	Number of repeats
P-1	IIE GLY GLY ASN LEU SER ALA	1
P-2	GLY VAL ALA IIE THR MET LYS	2
P-4	HIS SER THR PRO SER SER PRO	7
P-6	ASN ASP LEU MET ASN ARG ALA	2
P-8	ASP SER SER LEU PHE ALA LEU	3

✓ Three clones, P-4, P-6 and P-8 were chosen for further study

Identification of phage peptides specifically internalized by A20 cells

- Binding capacity of the candidate peptides to the A20 cells



	Sample Name	FL1 positive :: Freq. of Parent
	A20_P4-12uM008.fcs	87.2
	A20_P4-8uM006.fcs	67.6
	A20_P4-4uM007.fcs	37.4
	A20_US001.fcs	2.00

	Sample Name	FL1 positive :: Freq. of Parent
	A20_P6-12uM011.fcs	47.5
	A20_P6-8uM010.fcs	36.6
	A20_P6-4uM009.fcs	36.9
	A20_US001.fcs	2.00

	Sample Name	FL1 positive :: Freq. of Parent
	A20_P8-12uM004.fcs	82.6
	A20_P8-8uM003.fcs	63.2
	A20_P8-4uM002.fcs	45.2
	A20_US001.fcs	2.00

Fig. 1 Flow cytometry of the binding potential of Peptide-FITC conjugates for target cells. P4-, P6- and P8-FITC conjugates were incubated at 0, 4 or 8 μM with 10^6 A20 cells and analyzed for peptide binding. P4 and P8 peptides demonstrated a strong dose-dependent binding to the target A20 cells. These peptides were then tested for binding specificity by exposure to a series of off-target cells as shown in Fig. 3

Identification of phage peptides specifically internalized by A20 cells

- Confocal microscopy to compare peptide internalization into A20 cells

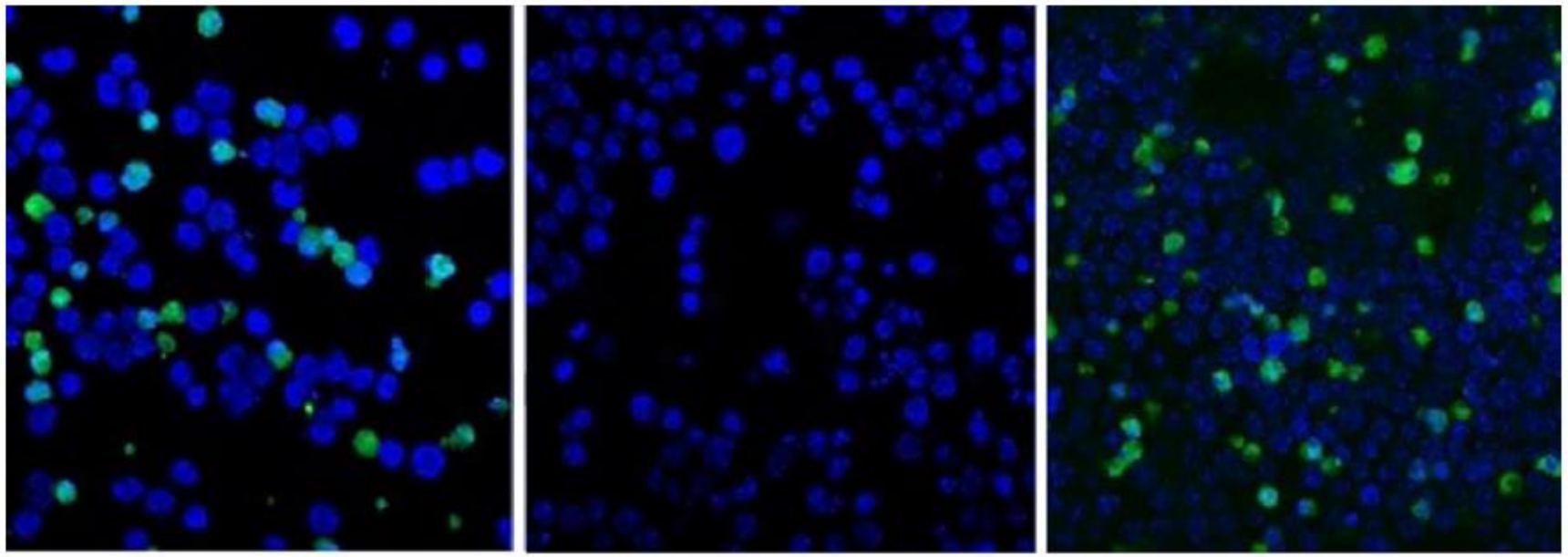


Fig. 2 Ability of peptides to internalize into A20 cells. P4, P6 and P8-FITC conjugates were incubated with A20 cells. After several washes and counterstaining with DAPI, the cells were viewed by confocal microscopy. The P6 conjugate did not penetrate into the cells unlike P4 and P8. The FITC marker localization is extra-nuclear

Specificity of peptides for target and off-target cells

- Based on the results of previous section and peptide copy numbers
- P4 as a positive binder and P6 as a negative/weak binder
- Testing by flow cytometry the P4-FITC and P6-FITC for their binding at several concentrations to a panel of off-target lymphoid and myeloid cells:

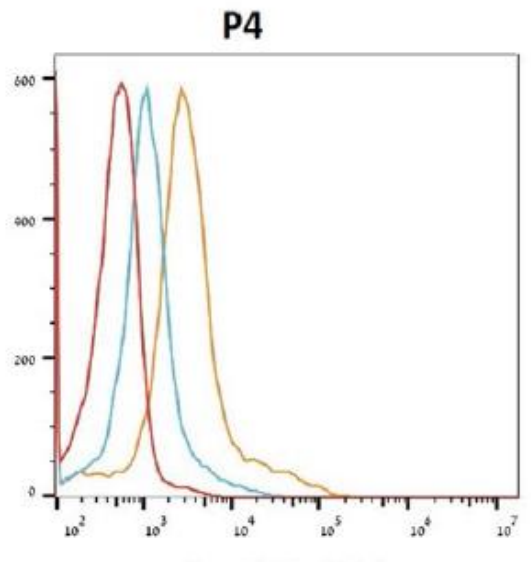
✓ PBMC leukemic cells
✓ Murine MOPC 315.BM leukemic cells
✓ HL-60 and NB-4

- Mouse peripheral mononuclear cells (PBMC), human leukemic cell lines (HL-60(human acute promyelocytic leukemia) and NB-4(human acute myeloid leukemia)

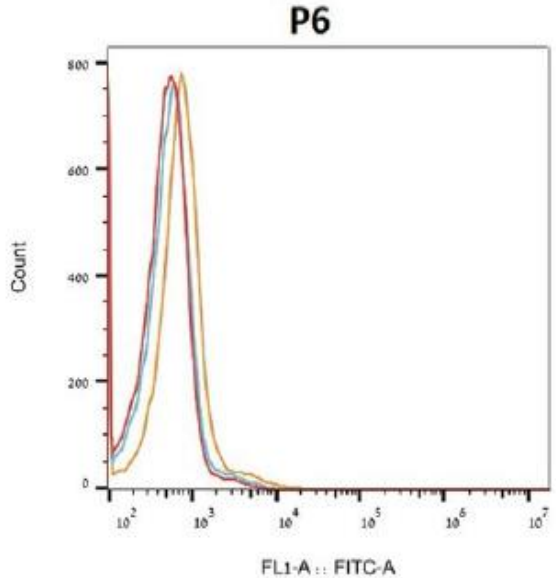
Specificity of peptides for target and off-target cells

- Flow cytometry analysis of P4 and P6 peptides binding to off-target cells (PBMC)

A20

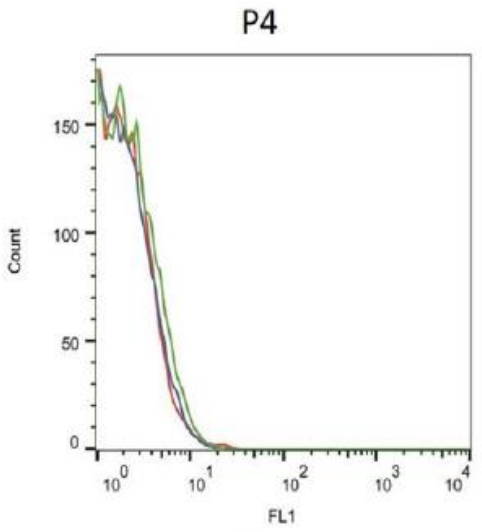


	Sample Name	Freq. of Parent	Workspace Annotation
	01-A20-D1.fcs	1.83	US
	01-A20-D2.fcs	25.5	P4-4uM
	01-A20-D3.fcs	85.9	P4-8uM



	Sample Name	Freq. of Parent	Workspace Annotation
	01-A20-D1.fcs	1.83	US
	01-A20-D4.fcs	3.57	P6-4uM
	01-A20-D5.fcs	7.15	P6-8uM

PBMC



	Sample Name	FITC positive :: Freq. of Parent
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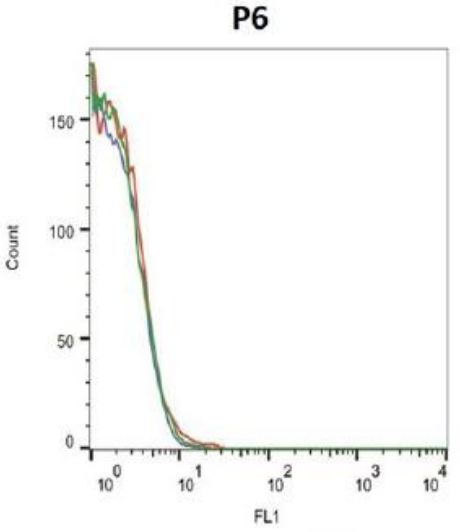
	PBMC mouse_US001.fcs	2.17

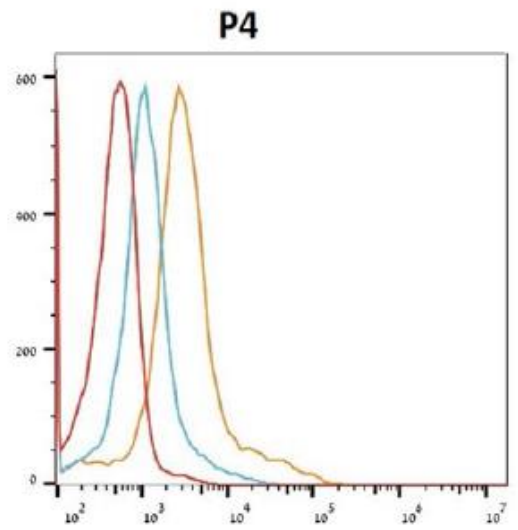


	Sample Name	FITC positive :: Freq. of Parent
	PBMC mouse_P6 8uM003.fcs	1.43
	PBMC mouse_P6 4uM002.fcs	0.86
	PBMC mouse_US001.fcs	2.17

Specificity of peptides for target and off-target cells

- Flow cytometry analysis of P4 and P6 peptides binding to off-target cells (MOPC 315.BM)

A20



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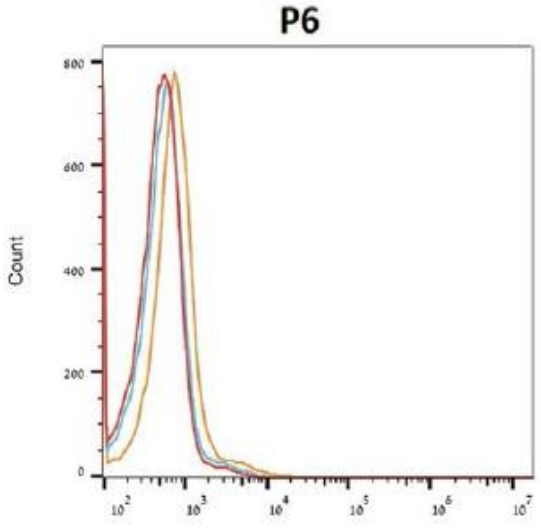
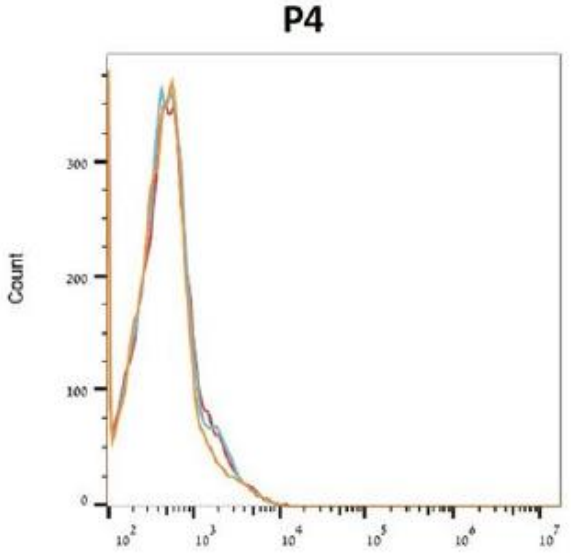
	01-A20-D2.fcs	25.5	P4-4uM
	01-A20-D3.fcs	85.9	P4-8uM



	Sample Name	Freq. of Parent	Workspace Annotation
	01-A20-D1.fcs	1.83	US
	01-A20-D4.fcs	3.57	P6-4uM
	01-A20-D5.fcs	7.15	P6-8uM

MOPC



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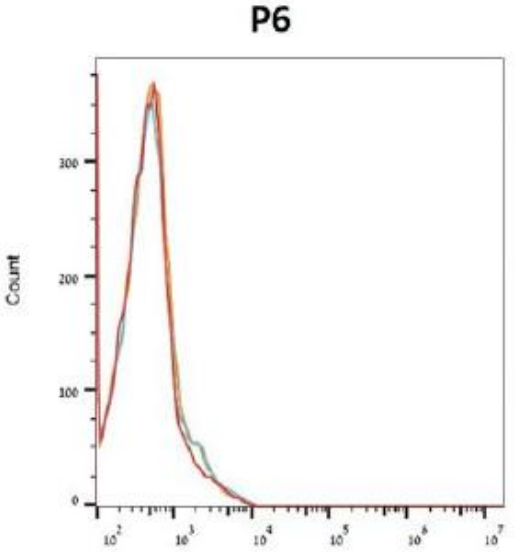

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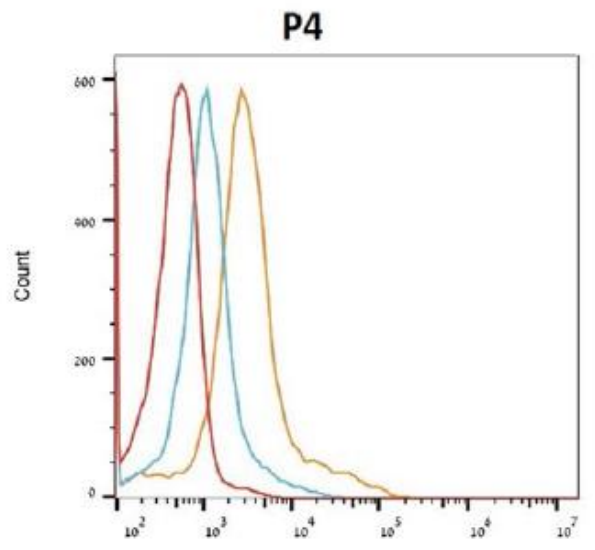
	01-MOPC-G4.fcs	0.50	P6-4uM
	01-MOPC-G5.fcs	0.93	P6-8uM

Specificity of peptides for target and off-target cells

- Flow cytometry analysis of P4 and P6 peptides binding to off-target cells (NB-4)

A20



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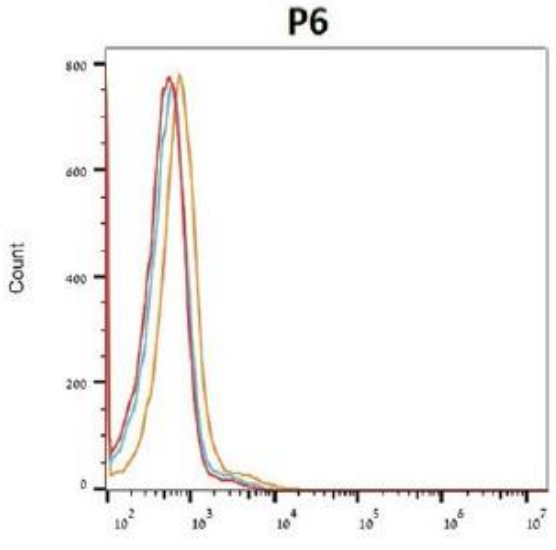
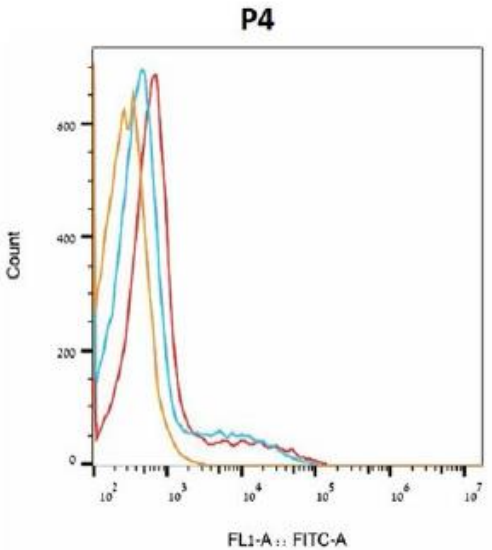
	01-A20-D3.fcs	85.9	P4-8uM



	Sample Name	Freq. of Parent	Workspace Annotation
	01-A20-D1.fcs	1.83	US
	01-A20-D4.fcs	3.57	P6-4uM
	01-A20-D5.fcs	7.15	P6-8uM

NB4



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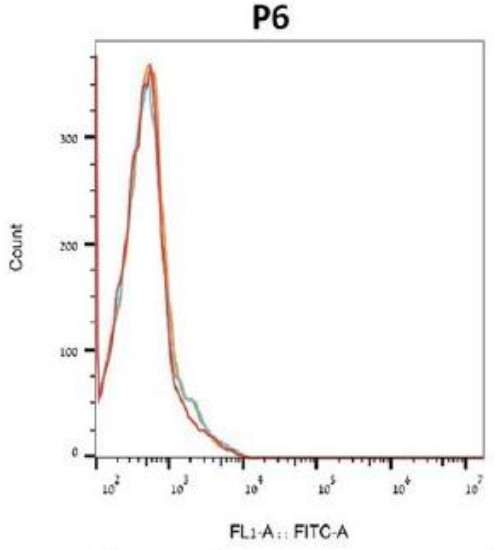
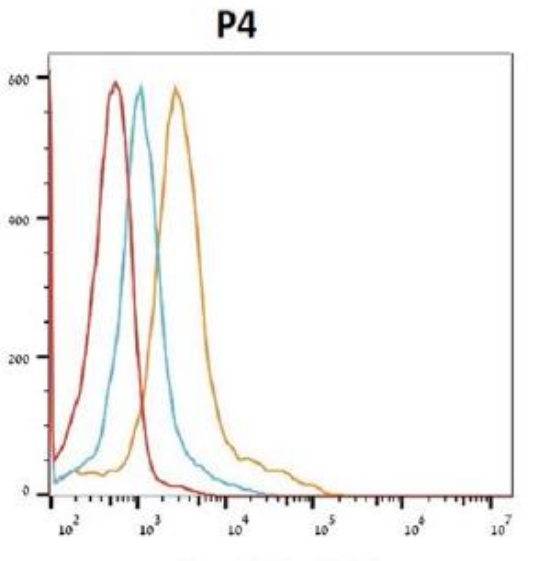


	Sample Name	Freq. of Parent	Workspace Annotation
	01-MOPC-G1.fcs	0.72	US
	01-MOPC-G4.fcs	0.50	P6-4uM
	01-MOPC-G5.fcs	0.93	P6-8uM

Specificity of peptides for target and off-target cells

- Flow cytometry analysis of P4 and P6 peptides binding to off-target cells (HL-60)

A20



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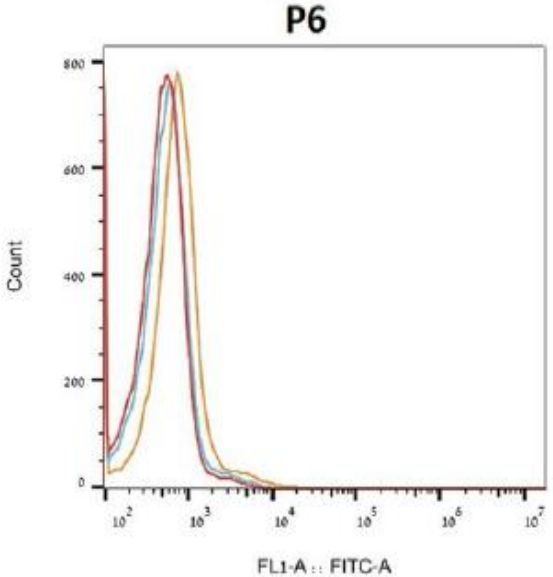
	01-A20-D2.fcs	25.5	P4-4uM
	01-A20-D3.fcs	85.9	P4-8uM



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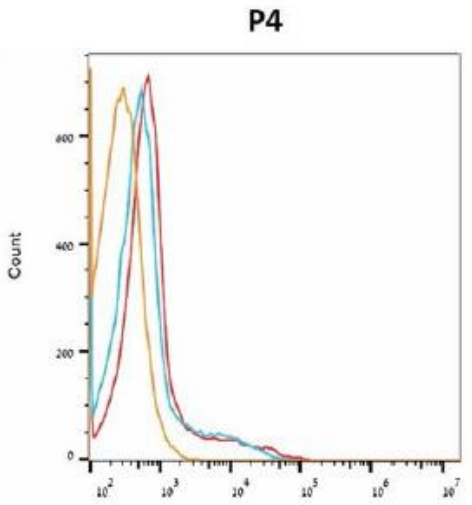
	01-A20-D5.fcs	7.15	P6-8uM

HL-60



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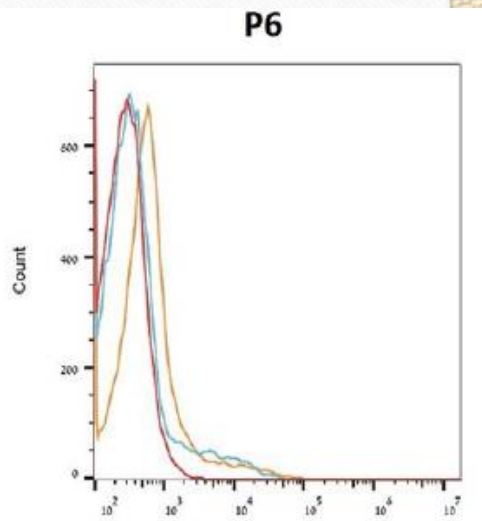

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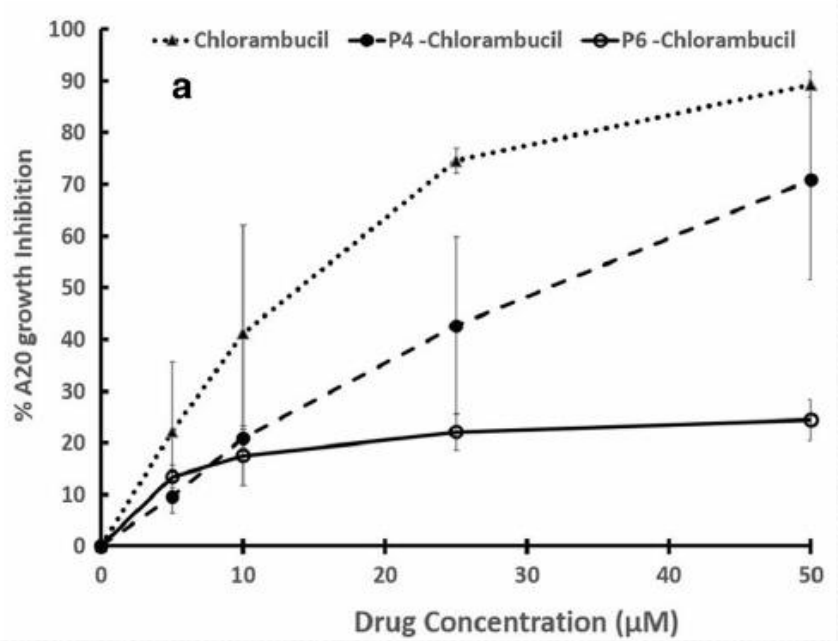
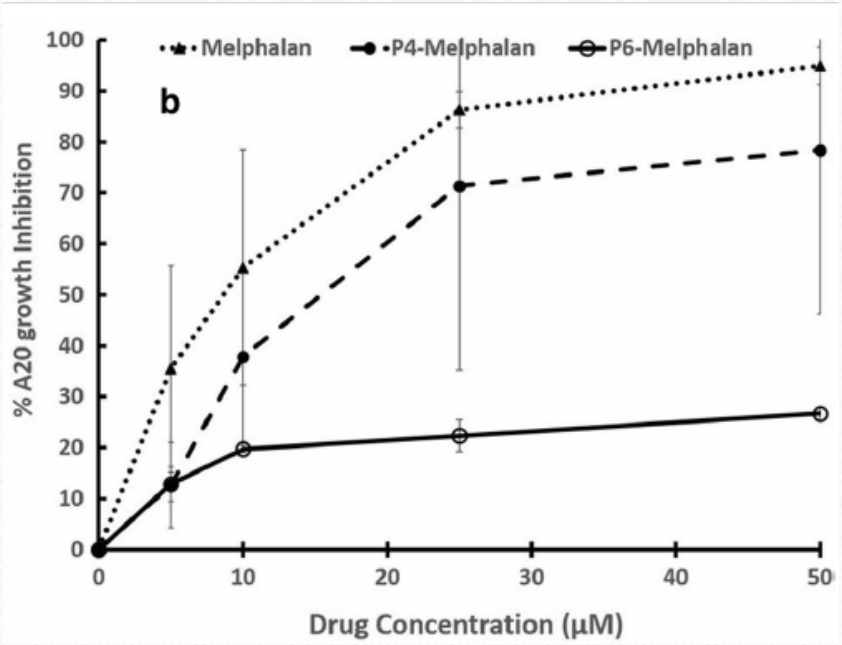
	01-HL60-F4.fcs	0.53	P6-4uM
	01-HL60-F5.fcs	6.92	P6-8uM

Cytotoxicity of free and peptide conjugated drugs

Cells were seeded overnight on microplate culture wells, washed, and re-cultured with fresh medium containing increasing doses (0–50 μM) of free chlorambucil, melphalan or bendamustine or P4- or P6-conjugates of these drugs. After a further 72 h culture, the cytotoxicity of the compounds was determined by measuring cellular metabolism (Fig. 4). The results were expressed as % Growth Inhibition compared to cells not exposed to drug. Clearly A20 cells are significantly more sensitive to Chlorambucil and Melphalan than they are to bendamus-

➢ Cytotoxic effect of free drugs or P4-PDCs against target cells





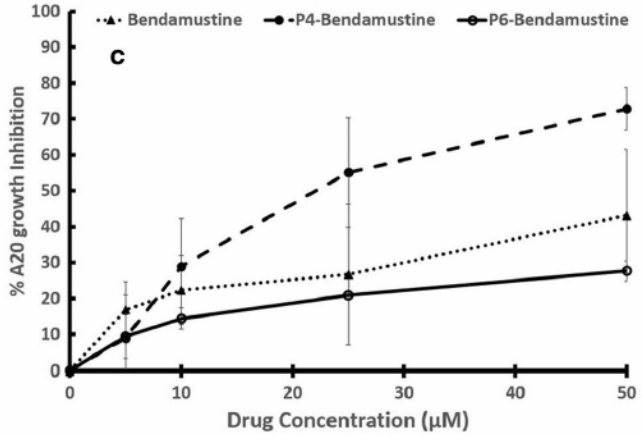
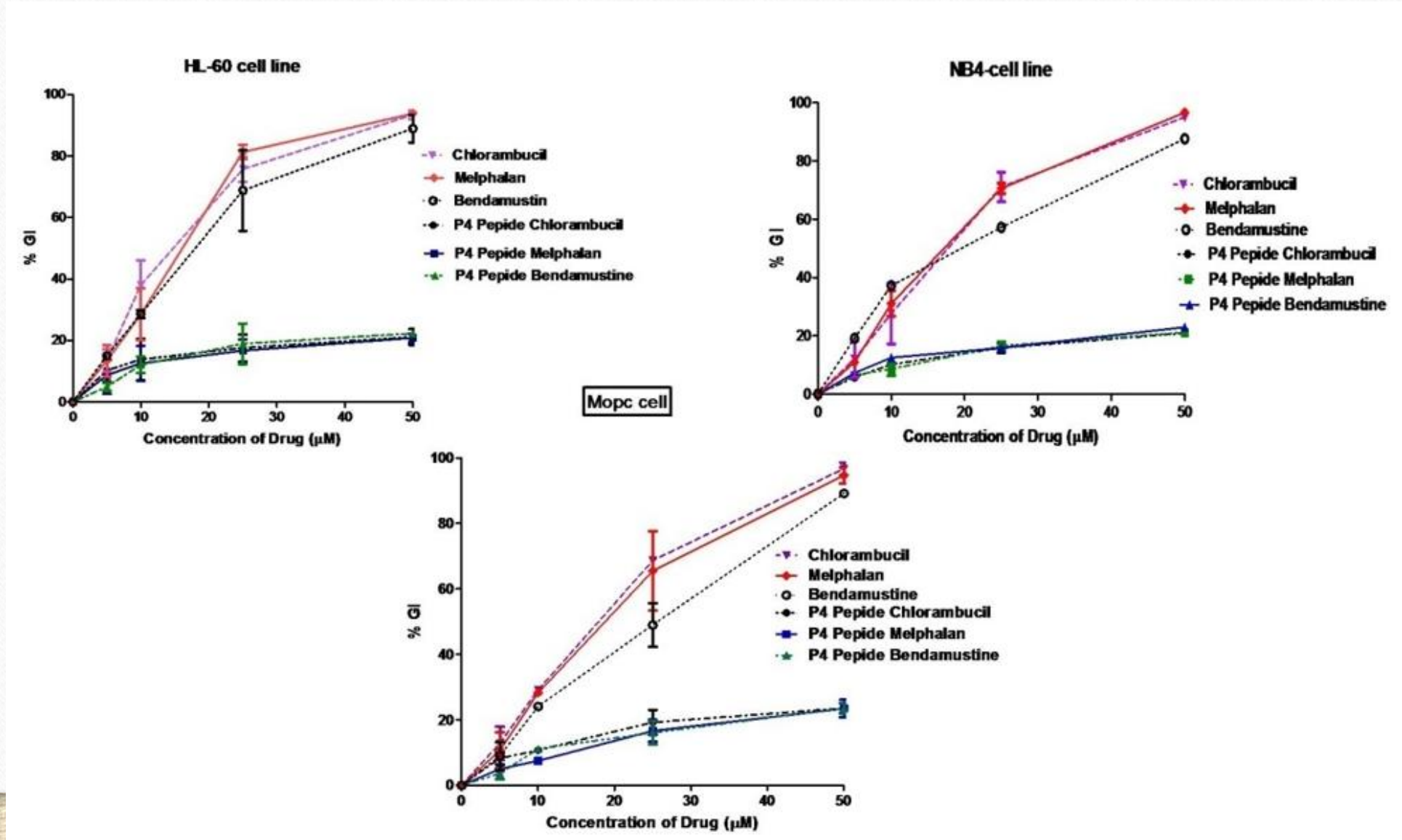


Fig. 4 Cell growth Inhibition assay by free and conjugated drugs. Growth inhibiting effect of drug-conjugates VS free drugs was studied on **a** chlorambucil, **b** melphalan, **c** bendamustine. At the end of 72 h incubation period cell growth was assessed using the XTT assay: optical density (OD) was measured at 480 and 680 nm—the latter is the background absorbance. The difference between the 480 and 680 nm measurement was used to calculate the % growth inhibition (GI) in test wells compared with control cells exposed to medium alone. The results shown for each concentration point represent the mean ± standard error for two independent experiments each conducted in (n = 3)

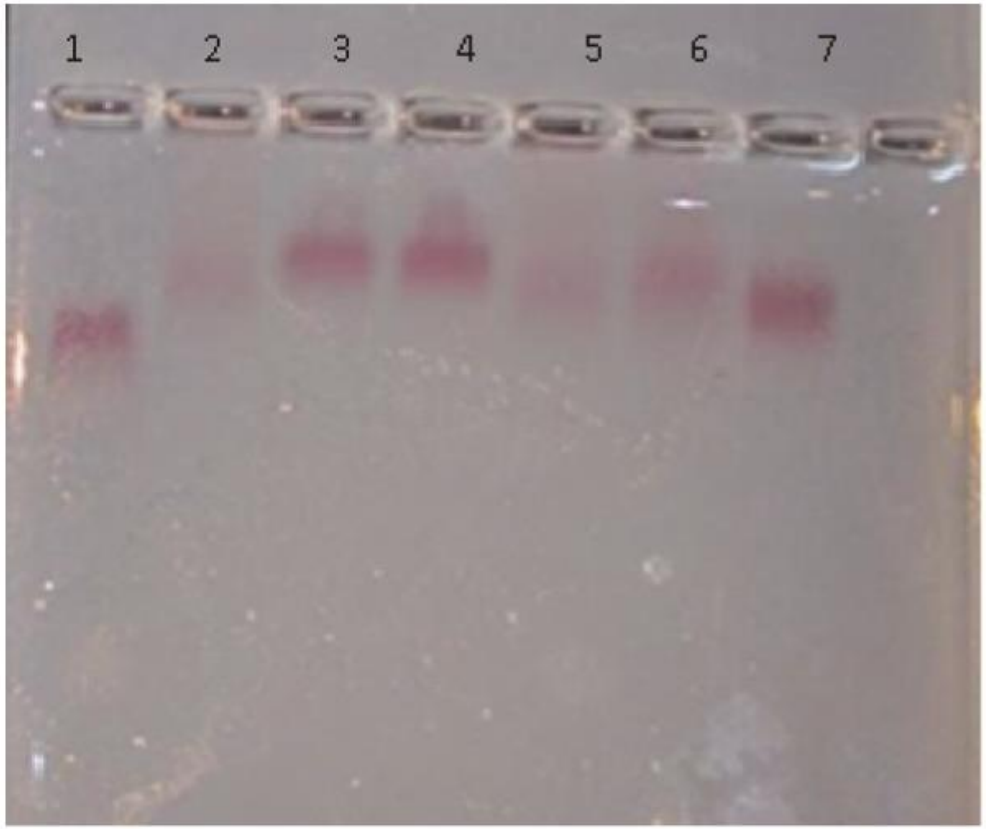
tine (IC_{50} values 15.8, 9.6, > 50 µM respectively; maximal %GI 89.4, 94.9 and 43.1 respectively). Conjugation of the drugs to P4 affected their efficacy toward A20 cells. For chlorambucil and melphalan, conjugation reduced the cytotoxic effect and this was significant for chlorambucil at 25 µM ($p = 0.0013$). On the other hand, conjugation significantly improved the cytotoxic effect of bendamustine at 25 ($p = 0.043$) and 50 µM ($p = 0.048$). The efficacies of all P6-conjugates were significantly lower than those of P4–conjugates at concentrations above 10 µM.

➢ Cytotoxic effect of free drugs or P4-PDCs against off-target cells



Characterization of PDC-coated PEG-AuNPs

- Electrophoresis mobility assay



Lane 1: PEG-6000 AuNP
Lane 2: PEG-6000 AuNP-P4–Chlorambucil
Lane 3: PEG-6000 AuNP-P4–Melphalan
Lane 4: PEG-6000 AuNP P4-Bendamustine
Lane 5: PEG-6000 AuNP P6-Chlorambucil
Lane 6: PEG-6000 AuNP P6-Melphalan
Lane 7: PEG-6000 AuNP P6-Bendamustine

Figure S4: Electrophoretic mobility of AuNPs and PDC-AuNPs. In a 1.5% agarose gel in TAE running buffer

Characterization of PDC-coated PEG-AuNPs

- **Size** and morphology of the PDC-PEG coated-Au nanoparticles (UV–VIS spectra analyses)

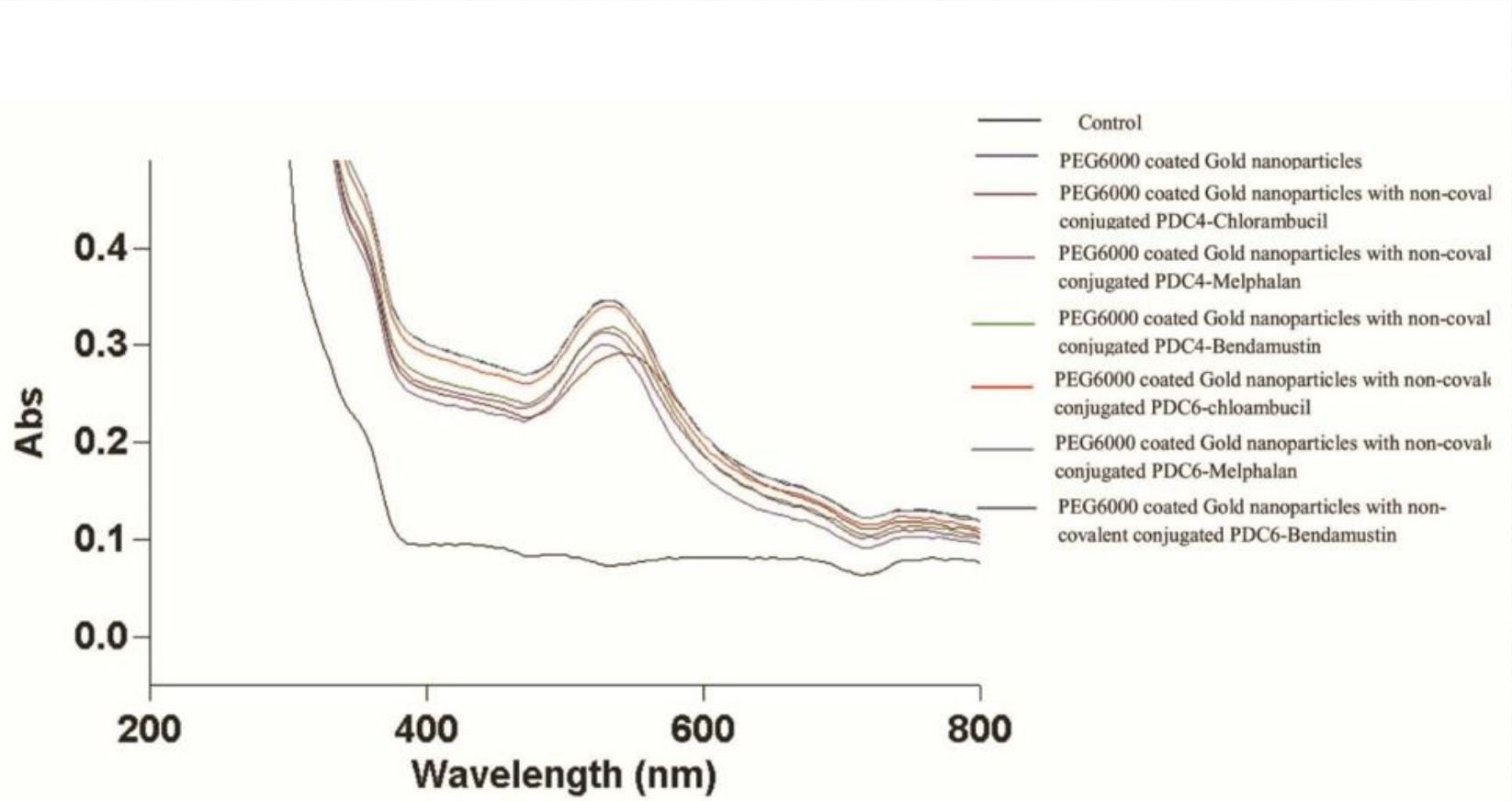


Figure S5: UV-VIS spectra in the range of 100-800nm of PEG-coated gold particles along and those non-covalently coated with peptide-drug-conjugates (PDCs)

Characterization of PDC-coated PEG-AuNPs

- Size and **morphology** of the PDC-PEG coated-Au nanoparticles (TEM analyses)

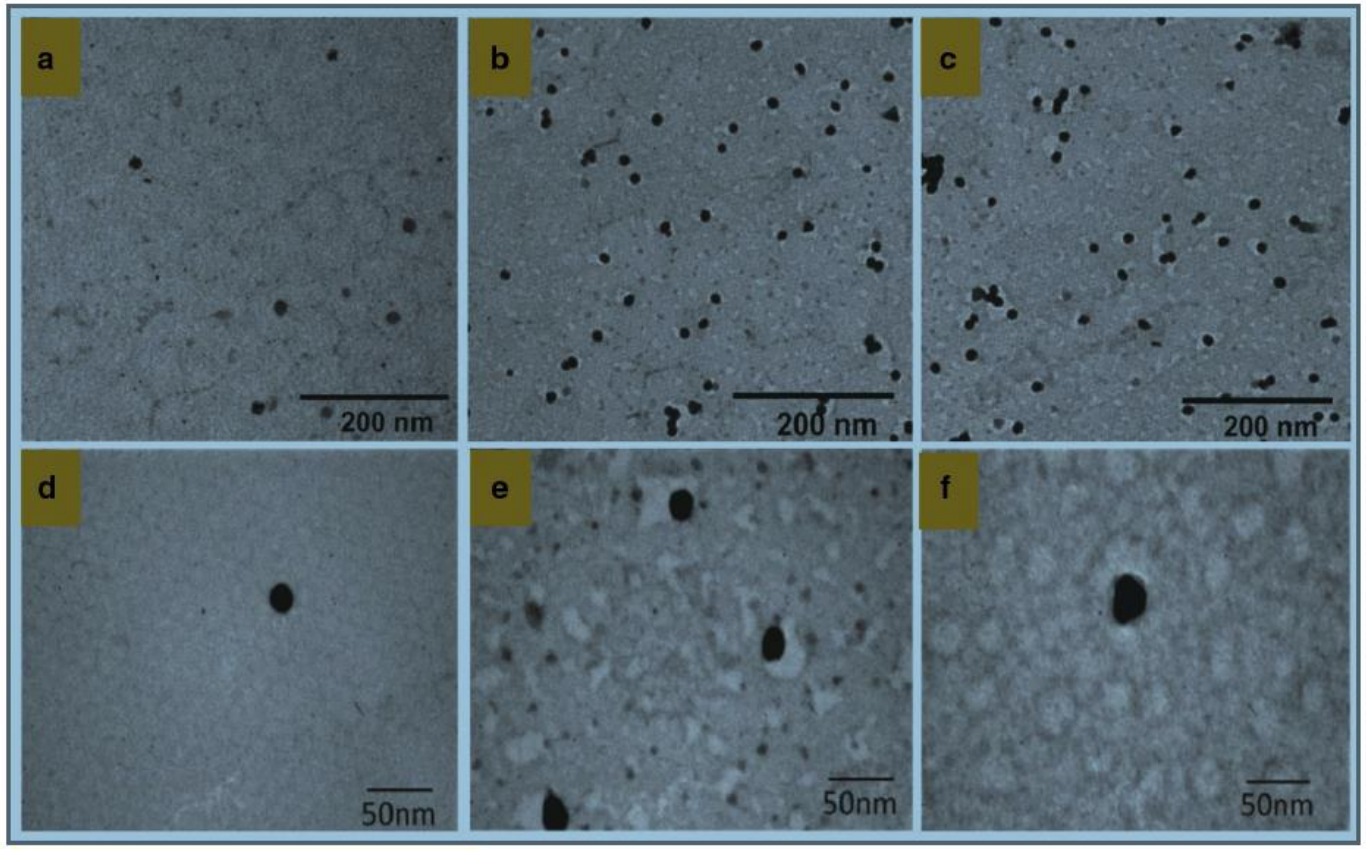


Fig. 6 TEM images of PEG-coated gold nanoparticles. Samples were negative stained with 1% phosphotungstic acid; thereafter, images were taken. Scale bars = 200, 20 nm. **a**, **d** Naked Gold nanoparticles (negative stained with 1% phosphotungstic acid; there is no white coated around the nanoparticles (uncoated PEG6000). **b**, **e** Gold nanoparticles coated PEG-6000 (negative stained with 1% phosphotungstic acid) black color gold nanoparticles—white color PEG6000 (negative stain). **c**, **f** Gold nanoparticles coated PEG-6000 + PDC4 – chlorambucil (negative stained with 1% phosphotungstic acid)

Characterization of PDC-coated PEG-AuNPs

- FTIR analyses of coated nanoparticles

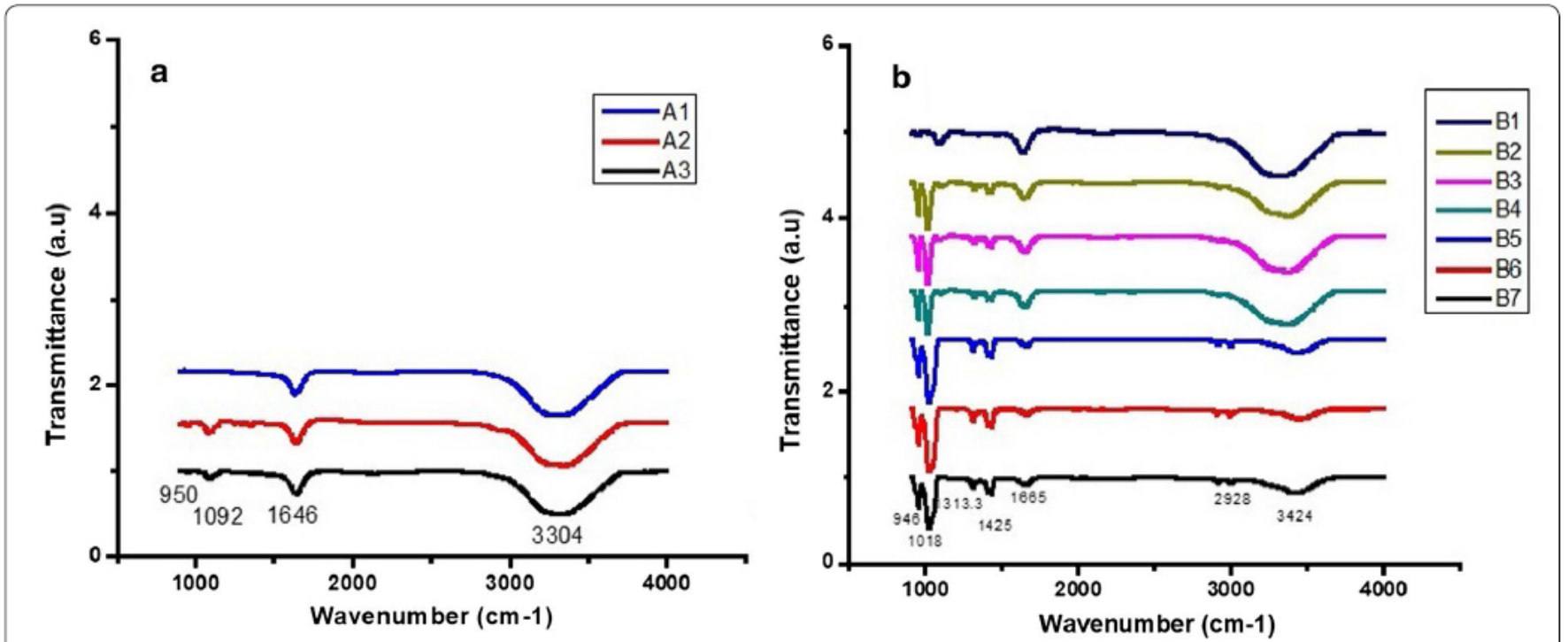


Fig. 7 Fourier Transformed Infra-Red spectra of PEG and PDC binding to gold nanoparticles. FTIR analysis was used to characterize the chemical binding of both PEG and Peptide drug conjugate to the surface of the nanoparticle. For the chemical bond significant of each peak, please refer to the text. **a** A1—gold nanoparticles; A2—PEG 6000; A3—PEG-6000 coated gold nanoparticles. **b** B1—PEG-6000 coated gold nanoparticles; B2—gold nanoparticles coated peptide4—chlorambucil; B3—PEG-6000 coated gold nanoparticles—PDC peptide4—melphalan; B4—PEG-6000 coated gold nanoparticles—PDC peptide4—Bendamustine; B5—PDC peptide4—chlorambucil; B6—PDC peptide4– melphalan; B7—PDC peptide4—bendamustine

Stability of P4-drug PDCs Vs stability of PDC coated PEG-AuNPs

- Chemostability

PDCs; the functions of these curves were used to calculate the t½ values. The results (Fig. 5a) showed that after 30 min incubation in buffer, 80% of PDCs had decomposed and they had completely broken down by 1.5 h. The t½ values ranged from 19.3 to 24.6 min (Additional

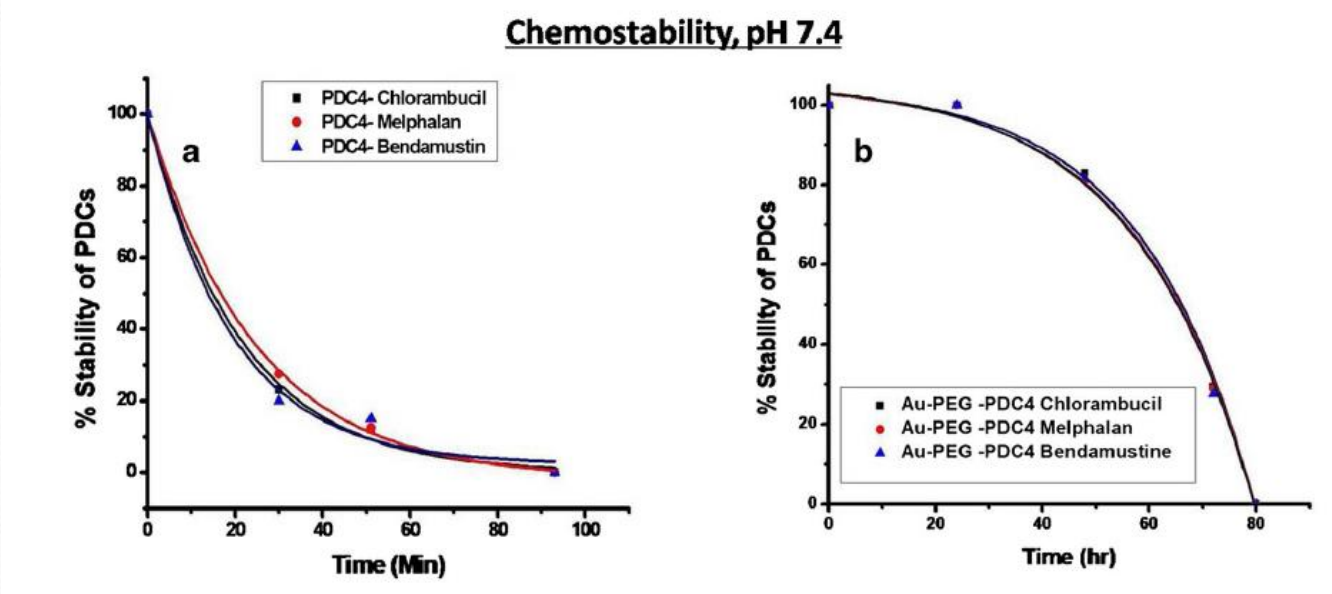
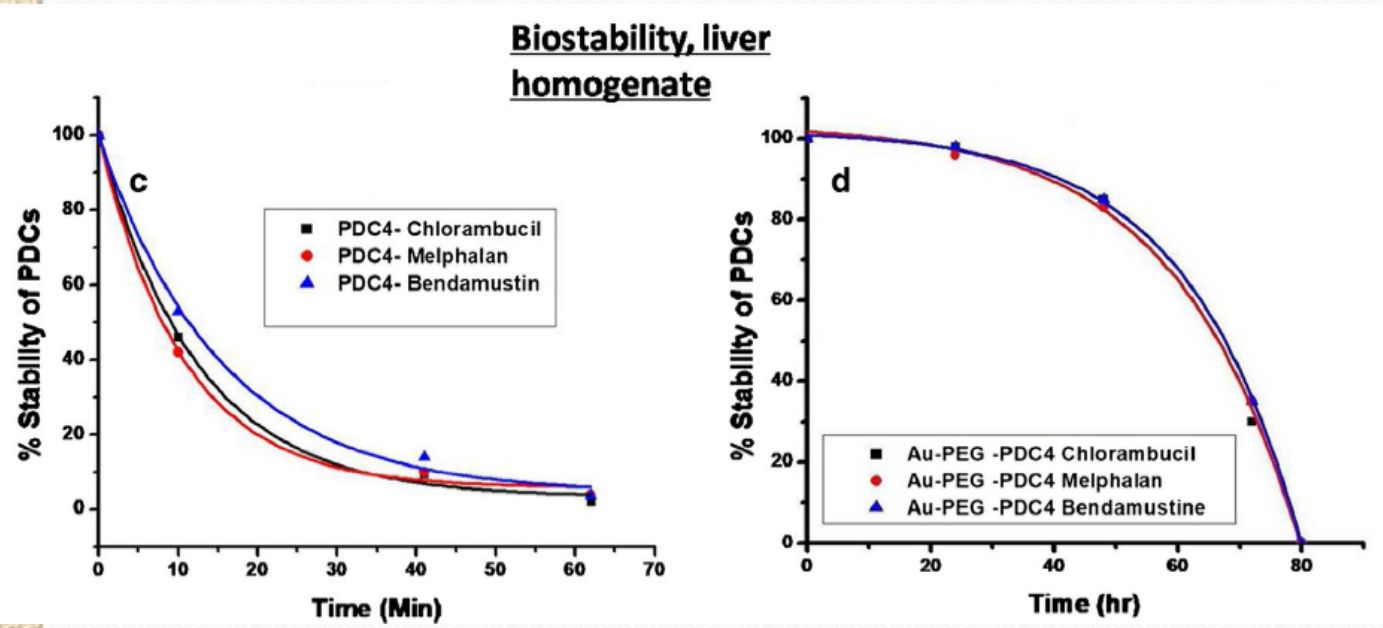


Fig. 5 Stability of free PDCs and PDC-PEG-AuNPs. Chemostability was assessed by incubation the constructs in 0.01 M phosphate buffer solution pH 7.2 at 37 °C for different time intervals. The pDC integrity was analyzed by LC–MS

Stability of P4-drug PDCs Vs stability of PDC coated PEG-AuNPs

- Biostability

✓ with half-lives ranging from **10.6 to 15.4 min**.



at physiological pH 7.4 was carried out. Aliquots were taken at various time points, and the concentrations of released drug were determined by quantitative LC–MS analyses. Figure 5b shows that the PDCs were completely stable over the first 24 h in contrast to the limited stability of PDCs alone (Fig. 5a). After 48 h stability had been reduced by 17% and by 100% after 80 h. The t½ values ranged from 21.0 to 22.3 h. There was no significant difference in behavior between the three PDC conjugates.

Fig. 5 Stability of free PDCs and PDC-PEG-AuNPs. Chemostability was assessed by incubation the constructs in 0.01 M phosphate buffer solution pH 7.2 at 37 °C for different time intervals. The pDC integrity was analyzed by LC–MS

Cytotoxicity of PDC-coated PEG-AuNPs

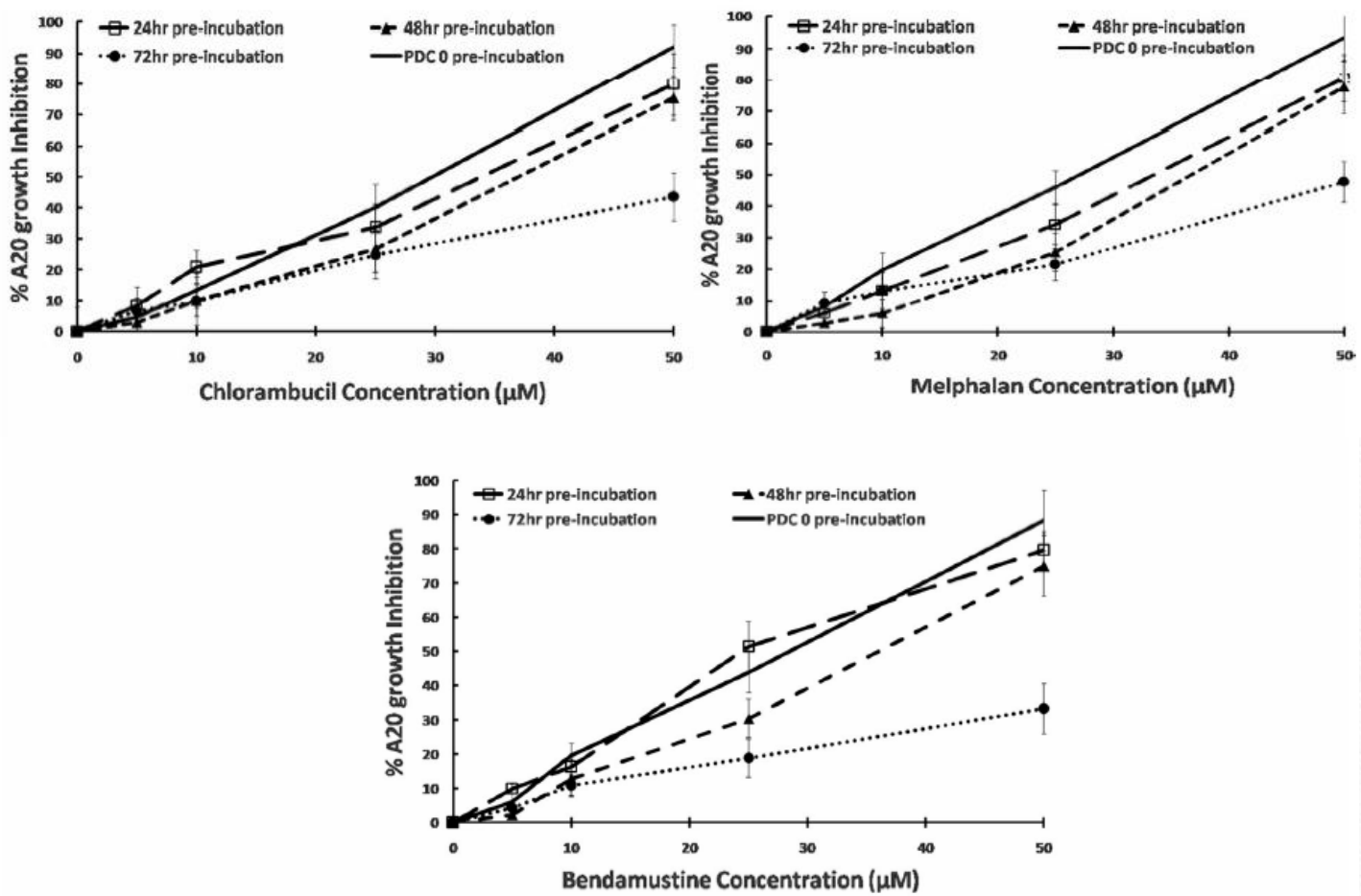


Fig. 8 Effect of drug or P4-PDC pre-incubation of cytotoxic activity. Free drugs, free P4-PDCs or P4-PDC-PEG-AuNPs were incubated for 24,48 or 72 h at 37 °C and then added to A20 cells for a further 72 h after which cell growth was assessed using the XTT assay: optical density (OD) was measured at 480 and 680 nm—the latter is the background absorbance. The difference between the 480 and 680 nm measurement was used to calculate the % growth inhibition (GI) in test wells compared with control cells exposed to medium alone. The results shown for each concentration point represent the mean ± standard error for two independent experiments each conducted in (n = 3). Only the results for fresh P4-PDC versus pre-incubated P4-PDC-PEG-AuNPs are shown as pre-incubation of free drugs or free PDC4 abolished their cytotoxic activity by more than 93%

the cytotoxic activity of the P4-PDC. Free drugs, free PDC4 and P4-PDC-PEG-AuNPs were pre-incubated in culture medium for 24, 48 or 72 h at 37 °C and then added to fresh A20 cells for a further 72 h. Figure 8 shows the results, corrected for cytotoxicity induced by gold particles alone (5–12% from lowest to highest concentration). All three P4-PDC-coated gold nanoparticles pre-incubated for 24 or 48 h induced statistically similar cytotoxicity in A20 to that induced by freshly prepared PDC4 and to coated particles without pre-incubation

Discussion

- One of the main goals of targeted drug delivery systems:
- Reduce unwanted toxicities against healthy cells
- Phage display peptide technology is a powerful tool for the discovery of novel peptides
- The stock phage pool contains many clones with low affinity for candidate target receptors
- lead to a cross-reactive or non-specific binding that y competes with less abundant, but higher affinity clones
- Removal the library clones with affinity for cell surface components present on several types of off-target cells
- Increasing the possibility of fishing out clones that bind unique receptor(s) on the A20 target cells

Discussion

- Discovering several clones internalized by the target cells using the “absorbed phage library”
- The P4, P6 and P8 were chosen to study
- The suitability of P4 as a candidate drug carrier
- Testing the cytotoxic efficacy of three chemotherapeutics
- Bendamustine enters the cell through the hOCT1
- Chlorambucil is taken up by simple diffusion
- Melphalan is actively taken up by LAT1

Discussion

Incorporation of the drugs into PDCs reduced the dose–response effect of Chlorambucil and Melphalan but significantly improved the effect of Bendamustine. These variations are to be expected given that PDC incorporation will depend on the receptor cell surface density and the mechanism of internalization. Indeed conjugation

mined that the loss of PDC integrity was mostly due to the hydrolysis of the two chlorines on all three nitrogen

Conclusions

- PDCs by possessing a number of chemical, structural and biological advantages
- Improving the targeted delivery and efficacy of chemotherapeutic drugs
- Limitation to their clinical application :the relatively short half-live
- Pegylated-gold nanoparticles significantly extending PDC stability

Future perspective

- The ability of AuNPs to extend the bioavailability of PDCs may:

  - Developing prolonged targeted cancer cell treatment protocols
  - Lead to slow release, targeted drug delivery systems
  - Application of gold nanoparticles in oncology, including hematological cancers by combining with the potential for TDD of PDCs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